Indonesian J. Pharm. Vol. 29 No. 4 : 214 – 221
ISSN-p : 2338-9427
DOI: 10.14499/indonesianjpharm29iss4pp221

Research Article

Efficacy of Thymol and Eugenol Against Polymicrobial Biofilm

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Submitted: 10-10-2018
Revised: 20-11-2018
Accepted: 25-11-2018

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ABSTRACT

Biofilms associated with human infection have high levels of pathogenicity due to their resistance to antibiotics. The discovery of an active antibiofilm agent against polymicrobial biofilms is a necessary consequence for coping with biofilm-related infections. Thymol and Eugenol are essential oils that have potential as antibacterial and antifungal. This study aimed to determine the effectiveness of thymol and eugenol inhibits *C. albicans, P. Aeruginosa, E. coli S. aureus* and polymicrobial biofilm. Biofilm formation inhibition assay and biofilm degradation assay of thymol and eugenol were determined using microtiter broth method.The antibiofilm efficacy of thymol and eugenol towards polymicrobial biofilms were analyzed by calculating minimum biofilm inhibitor concentration ($MBIC_{50}$) and minimum biofilm eradication concentration ($MBEC_{50}$) values. The data were analyzed using *Statistical Package for the Social Sciences* (SPSS) with 95% confidence level. Thymol and eugenol showed inhibitory activity against the formation of mono and polymicrobial biofilms of the microbial tested.The result also demonstrated an evidence of activity of thymol and eugenol in breaking down mono and polymicrobial biofilm. Therefore, thymol and eugenol serves as a potential source for new antibiofilm drugs towards polymicrobial biofilm.

Key words: Thymol, Eugenol, polymicrobial biofilm, $MBIC_{50}$, $MBEC_{50}$

INTRODUCTION

Biofilm-related infections are an increasing health problem worldwide, especially patients suffering from immune system disorders such as HIV, cancer, organ transplants and malnutrition. Biofilm associated with human infection may contribute tomicrobial resistance towards antibiotic used. It is estimated that 65% of all hospital-related infections is caused by biofilms which includes its attachment on medical devices such as catheters and biomaterials (Furukawa *et al*., 2006). Biofilm in nature consists of mixture of various strain and even various species of microbes living together. The microbial diversity in polymicrobial biofilms adds complexity in the eradication process (Liu *et al*., 2000).

Many recent research on biofilm eradication have been done on single species microbial biofilms. However, polymicrobial biofilms can cause chronic infections while synergistic interactions in of the polymicrobials affects the physiological function of biofilms by resulting increased resistance and virulence (Burmølle*et al*., 2014). The formation of biofilms from both bacterial polymicrobials (Gram positive and Gram negative) as well as fungi especially from the genus *C. albicans* is responsible for the onset of disease in humans. Some literature explain that bacteria synergistically can form biofilms with other bacterial species, and physically and physiologically the structure of the biofilm becomes thicker and stronger (Andersson*et al*., 2008; Cowan *et al*., 2003; Leriche *et al*., 2003).

Thymol and Eugenolare both found in plant essential oils. Thymol has a broad spectrum antimicrobial activity, and has been the subject of several studies *in vitro* (Dorman and Deans, 2000).Thymol and eugenol had activity inhibit mono-species biofilm *S. aureus* and *E. coli* (Nestro *et al*., 2007; Kim *et al.,* 2016). Despite being potential as antibiofilm against single species microbes, no reports on their effects eugenol against polymicrobial biofilms consists of *P. aeruginosa, E. coli*, *S. aureus*, *C.*

albicans. Research on such topic is essential to evaluate the effectiveness of eugenol and thymol, as antibiofilm in polymicrobial cultures.

MATERIALS AND METHODS

Materials used were thymol (Sigma-Aldrich, Germany), eugenol (Sigma-Aldrich, Germany), crystal violet (Merck, Germany), ethyl acetate (Merck, Germany), *Brain Heart Infusion* (Oxoid) (Merck, Germany), ethanol 95% (Merck, Germany), nystatin (Sigma-Aldrich, Germany), chloramphenicol (Sigma-Aldrich, Germany).

Equipments

Some equipments used in this research were *Laminar Air Flow*, incubator (IF-2B) (Sakura, Japan), *micropipette pipetman* (Gilson, France), *multichannel micropipette* (Socorex, Swiss), *microplate flat-bottom polystyrene 96 well* (Iwaki, Japan), *mikrotiter plate reader* (Optic Ivymen System 2100-C, Spain*),* spectrophotometry (Genesys 10 UV Scanning, 335903) (Thermo Scientific Spectronic, USA), *autoclave* (Sakura, Japan), analytical scales (AB204-5, Switzerland).

Bacterial Strains

A standardstrain of *Saureus* ATCC 25923*, Ecoli* ATCC 25922*, Paeruginosa* ATCC 27853*,* wascultured in tryptic soy broth (TSB) medium and incubated at 37ºC for 72h. *Calbicans* ATCC 10231 was cultured in Sabouraud Dextrose Broth (SDB) medium and incubated at 37ºC withagitation (120rpm) for 24h. The optical densities (OD_{600}) of microbial cultures will be adjusted to 0.1 (equal of the 0.5 McFarland standard ~$1.5x10^8$ CFU/mL), and subsequently diluted in fresh medium to OD_{600} 0.01 for each microbial species.

Biofilm Formation Inhibition and Biofilm Eradication Assay *in Vitro*

In order to determine the isolate eugenol and thymol activity toward formation and degeneration of biofilm, a *microtiter plate polystyrene flat bottom 96-well* was used (Pierce *et al.,* 2008). A 100µL of *C. albicans* suspension (10^7 CFU/mL) was inserted in each well *microtiter plate*, *C. albicans* were incubated at 37ºC for 90min for biofilm-adhesion phase. After the incubation, plates were washed using 150µL of sterile aquadest for three times in order to nullify the non-adhesive cells. A 100µL of media contained eugenol and thymol with different concentrations (1% v/v, 0.5% v/v, 0.25% v/v, 0,125% v/v), was added to each washed-well. Media with methanol were used as diluting controland microbe suspensions were used as negative control. As the positive control, a microbe suspension with nystatin 1% was used, and a media without microbe growth was used as media control. The plates were incubated in 37°C for 24h to form the mid-phase biofilm and 48h for maturating phase. Plates were washed with distilled water for three times. A 125µL solution of crystal violet 1% was added to each well in order to color the formed biofilm. The plates were incubated in a room temperature for 15min. After incubation, the microplates were washed with running water for three times to clean the crytal violet's remains and A 200µL of ethanol 96% was added to each well. The *Optical Density* (OD) reading was performed with microplate reader on the wavelength of 595nm. The testing was performed in three times replication. The percentage of inhibition and degeneration for every eugenol and thymol concentration was counted using the formula below:

$$\frac{\textbf{Od growth control – Od sample}}{\textbf{Od growth control}} \textbf{ x 100}$$

The amount of sample that could inhibit at least 50% of the biofilm formation can be considered as minimum biofilm inhibitory concentration ($MBIC_{50}$) and minimum biofilm eradication concentration ($MBEC_{50}$) (Pratiwi *et al* 2015). The testing for polymicrobial biofilm *C. albicans, E. coli, P. aeruginosa* and *S. aureus* was performed under the same methods with the monospecies *C. albicans*. In other hand, the biofilm testing towards *E. coli, P. aeruginosa* and *S*. aureus was similar to the monospecies testing without incubation of *C. albicans* at ±37ºC for 90min for adhesion phase. A microbial suspension that already given an antibiotic namely chloramphenicol 1% v/v, was used as control-antibiotic.

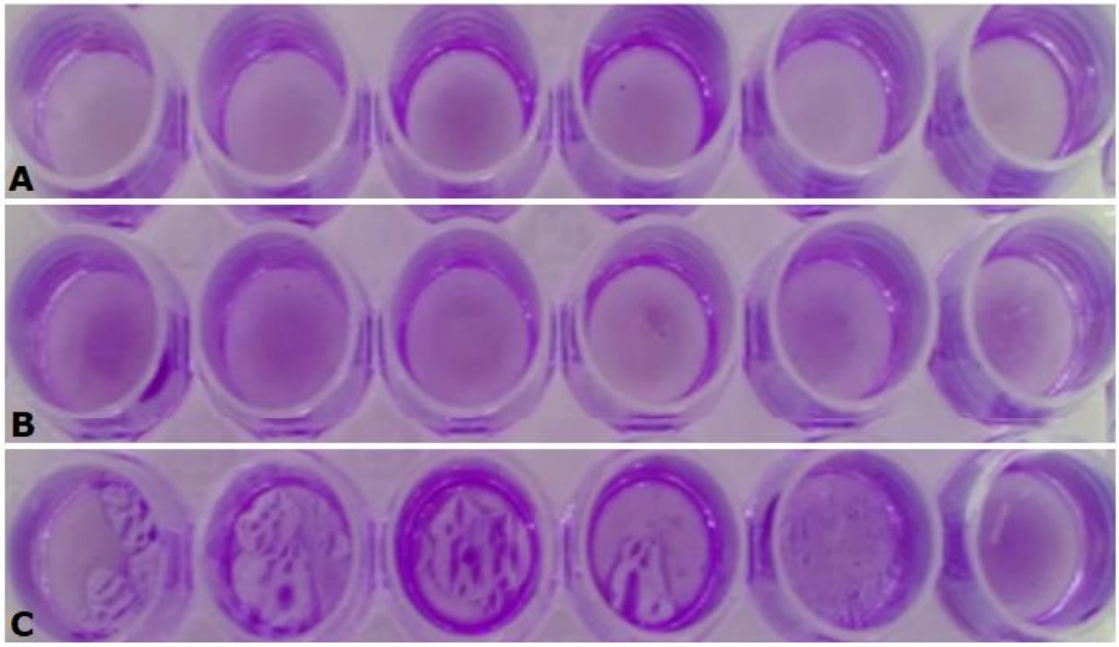

Figure 1. Crystal violet assay to see the formation of biofilm, a. Polymicrobial biofilm by adminitration of eugenol compounds; b. Polymicrobial biofilm by adminitration of thymol compounds; c. Polymicrobial biofilm without adminitration of test compound

RESULT AND DISCUSSION

Crystal violet assay for staining biofilm

Crystal violet staining is commonly used for quantification of biomass biofilm formation in various types of microorganisms. Crystal violet binds the negative charge on the surface of the molecule and polysaccharide to EPS so that crystal violet remains attached to the biofilm after being washed with distilled water (Peeters *et al.,* 2008). Crystal violet was used to show the quantity of biofilm mass (Stepanovic *et al.,* 2000). Crystal violet is bound to molecular surface negative ions and polysaccharides present in the extracellular matrix (Li *et al.,* 2003).

Biofilm formation can be seen in the microplate base after administration of crystal violet (Figure 1). Figure 1.C shows that the intensity of crystal violet staining is very clear, this showed that the composition of biofilm numbers located in these wells is very thick and strong where the biofilm is not well formed.

Whereas in figure 1.B the intensity of crystal violet staining is not as close as in Figure 1c.This indicates that biofilm formation is not completely formed. This inhibiting mechanism is caused by thymol compounds that inhibit polymicrobial biofim. Therefore, one bacterium was unable to form a community with other bacteria.

In figure 1. A, the intensity of staining of violet crystals is very low indicating that the biofilms formed are less than in figure 8.C and 8.B. This showed the inhibition of biofilm formation because the eugenol compound can inhibit the formation of *S. aureus*, *P. aeruginosa*, *E. coli* and *C. albicans* to form complex biofilm communities.

Effectof thymol and eugenol against mono-speciesmid – phase biofilm (24h)

In this study we evaluated the antibiofilm potencies of thymol and eugenol, each against polymicrobial and mono-species biofilms. We observed a decline in the rate of biofilm formation along with the increased concentrations of thymol and eugenol. The formation of *C. albicans* biofilm begins with the attachment of cells to host cells which last between 0-2h continuing with hyphae formation (4-6h). The hyphae yarns form a monolayer (6-8h) that will proliferate (8-24h) to then maturate (24-48h) (Ramage *et al*., 2001).

Table I. Effect of thymoland eugenol against mono-species mid – phase biofilm (24h)

Compound	Bacterial Strains	% Inhibition	$MBIC_{50}$ v/v
Thymol 1%	*P. aeruginosa*	84.10±1.26*	0.13%
	E. coli	85.30±0.52*	0.29%
	C. albicans	84.40±1.85*	0.20%
	S. aureus	59.70±0.90*	0.51%
Eugenol 1%	*P. aeruginosa*	86.86±1.15*	0.51%
	E. coli	82.87±0.62*	0.054%
	C. albicans	81.73±1.45*	0.06%
	S. aureus	57.56±0.51*	0.206%
Chloramphenicol 1%	*P. aeruginosa*	88.76±0.73*	
	E. coli	77.95±0.83*	
	S. aureus	59.02±1.10*	
Nystatin 1%	*C. albicans*	70.67±0.36*	

Table II. Effect of thymol and eugenol against mid – phase polymicrobial biofilm (24h)

Compound	% Inhibition	$MBIC_{50}$ v/v
Thymol 1%	84.05±0.25*	0.10%
Eugenol 1%	89.20±0.25*	0.31%
Chloramphenicol 1%	69.82±1.96*	
Nystatin 1%	55.70±0.72*	

The results of this study showed that the formation of mid-phase biofilms of *P. aeruginosa, E. coli, C. albicans* and *S. aureus* decreased with increasing concentrations of thymol compounds (Table I and Figure 2). Eugenol showed inhibitory activity against mid-phase mono-species and polymicrobials biofilm. The formation of mid-phase biofilms of *P. aeruginosa, E. coli, C. albicans* and *S. aureus* decreased with increasing concentrations of eugenol compounds (Table I and Figure. 3).

Effect of Thymol and Eugenol against mid – phase polymicrobial biofilm (24h)

The biofilm tested consisted of Gram-positive bacteria i.e *Staphylococus aureus* and Gram-negative bacteria i.e *Pseudomonas aeruginosa* - *Escherichia coli* and yeast *Candida albicans*. Previous study found that bacteria synergistically can form biofilms with other bacterial species that difficult to penetrate by antibiotics compared to planktonic cells (Andersson *et al*., 2008). EPS matrices in biofilms may help cells survive longer by providing protection layers against antibiotics (Anderson *et al*., 2008; Leriche *et al*., 2003).

Thymol 1% and eugenol 1%, each showed higher activity against Nystatin and Chloramphenicol of the same concentration. It was observed a concentration dependent antibiofilm activity towards a mid-phase polymicrobial biofilm (Table II and Figure 4). This value showed that the power of inhibiting activity of thymol and eugenol compound was strong and had potential as anti-biofilm medication's candidate.

Effect of Thymol and Eugenol against mono-speciesbiofilm maturation phase (48h)

In the maturation phase, antimicrobial agents will have more difficulties to penetrate the biofilm and eugenol compounds have the potential to be antibiofilm. We observed decreasing in biofilm growth with increasing concentration of thymol and eugenol compounds, as described in (Table III, Figure 5 and 6).

Table III. Effect of thymol and eugenol against mono-species biofilm maturation phase (48h).

Compound	Bacterial Strains	% Inhibition	$MBIC_{50}$ v/v
Thymol 1%	*P. aeruginosa*	84.82±0.30*	0.22%
	E. coli	80.34±0.78*	0.45%
	C. albicans	72.63±1.41*	0.43%
	S. aureus	55.21±1.70*	0.52%
Eugenol 1%	*P. aeruginosa*	84.36±1.05*	0.26%
	E. coli	74.50±0.60*	0.51%
	C. albicans	74.91±0.55*	0.51%
	S. aureus	56.31±0.41*	0.62%
Chloramphenicol 1%	*P. aeruginosa*	79.66±0.20*	
	E. coli	81.84±0.56*	
	S. aureus	65.31±0.78*	
Nystatin 1%	*C. albicans*	71.49±0.41*	

Table IV. Effect of thymol and eugenol against polimicrobial biofilm phase (48h).

Compound	% Inhibition	$MBIC_{50}$ v/v
Thymol 1%	82.27±0.36 *	0.26%
Eugenol 1%	71.66±0.58 *	0.50%
Chloramphenicol 1%	74.72±0.26 *	
Nystatin 1%	50.98±3.71 *	

Thymol caused 50% damage in maturation phase of the mono-species *P. aeruginosa, E. Coli, C. albicans* and *S. aureus*. Antibiotic (chloramphenicol) and antifungal (nystatin 1%) that used as control showed a lower biofilm inhibition compared to thymol and eugenol compound. In biofilm inhibition of mono-species *P.aeruginosa* and *C. albicans*, thymol 1% showed higher activity against Chlorampenicol and nystatin of the same concentration (Table III).

In this phase, the microbes that formed biofilm were already adhesive to the substrate, so those thymol and eugenol compounds were harder to kill the biofilm compared to the mid-phase. In this phase, microbes were forming a strong biofilm defense system, and built a communication cell mechanism called quorum sensing. These made the antibiotics were hard to kill and damage the bacteria that formed biofilm.

Effect of Thymol and Eugenol against polimicrobial biofilm phase (48h)

Our results in figure 7 showed that thymol and eugenol with 0.5% concentration can damage the polymicrobial biofilm defense system for 50 %. On the concentration of 1% thymol and eugenol had inhibiting activites for 82.27±0.36% and 71.66±0.58%, while in antibiotic (chloramphenicol) and antifungal (nystatin 1%) showed a smaller inhibiting activity (Table IV). We reported that the eugenol activity on maturation phase of the polymicrobial biofilm was better compared to chloramphenicol. This showed that eugenol is potential to be developed and improved as the candidate for anti-biofilm. Eugenol at sub-inhibitory concentrations inhibited the produ-ction of virulence factors, including violacein, elastase, pyocyanin, and biofilm formation (Zhou *et al.*, 2013)

$MBIC_{50}$ values for polymicrobial biofilm maturation phase were 0.26% v/v (Thymol) and 0.50% v/v (Eugenol). This value showed that thymol and eugenol compounds had strong inhibiting activity, but eugenol $MBIC_{50}$ value on the middle-phase was better compared to the maturation phase, because in the maturition phase, the heterogeneous pattern was found in the biofilm, that interacted one and another and had more complex structure.

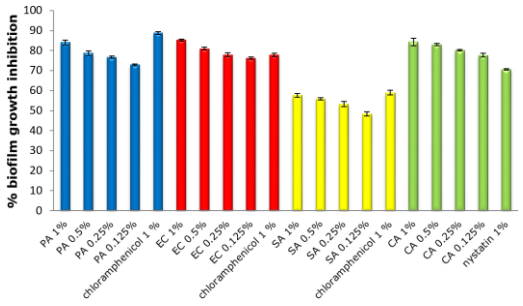

Figure 2. Effect of thymol against mono-speciesmid – phasebiofilm. Blue= *P. aeruginosa,* Red= *E.coli,* Yellow= *C.albicans,* Green= *S. aureus*

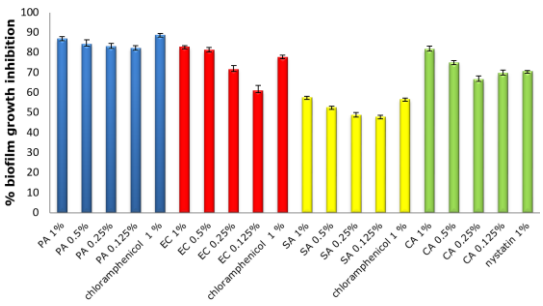

Figure 3. Effect of eugenol against mono-speciesmid – phase biofilm. Blue= *P. aeruginosa,* Red= *E.coli,* Yellow =*C.albicans,* Green= *S. aureus.*

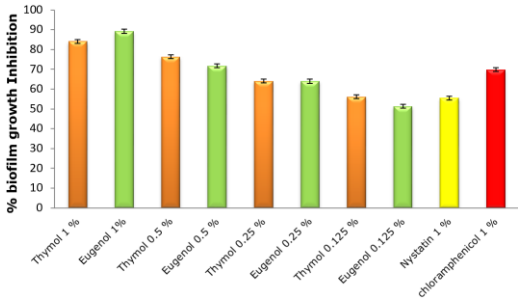

Figure 4. Effect of thymol and eugenol against polymicrobial mid – phasebiofilm. Brown = *Thymol,* Green= *Eugenol,* Yellow=*Nystatin,* Red= *Choloramfenicol.*

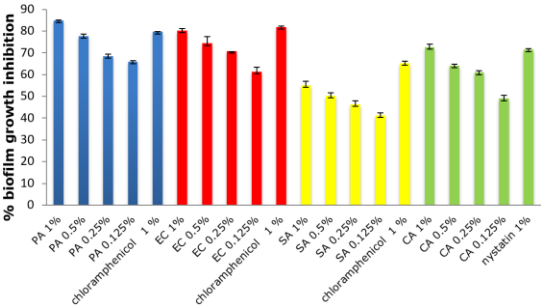

Figure 5. Effect of Thymol against mono-species maturation biofilm. Blue = *P. aeruginosa,* Red =*E. coli,* Yellow =*C. albicans,* Green = *S. aureus.*

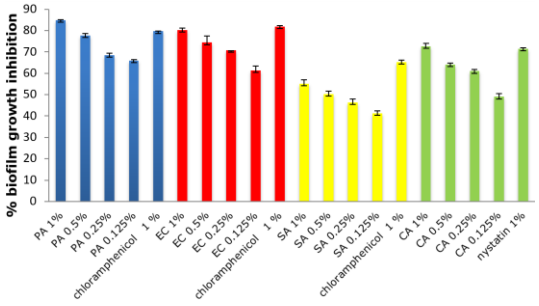

Figure 6. Effect of eugenol against mono-species maturation biofilm. Blue= *P. aeruginosa,* Red=*E. coli,* Yellow=*C. albicans,* Green = *S. aureus.*

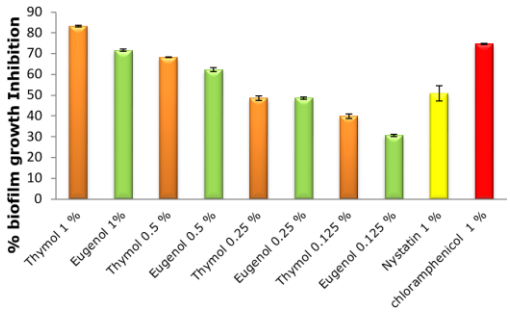

Figure 7. Effect of thymol and eugenol against polymicrobial maturation biofilm. Brown = *Thymol,* Green= *Eugenol,* Yellow =*Nystatin,* Red = *Choloramfenicol.*

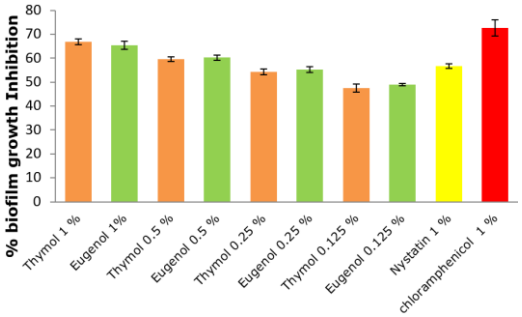

Figure 8. Effect of thymol and eugenol against polymicrobial eradication biofilm. Brown = *Thymol,* Green= *Eugenol,* Yellow =*Nystatin,* Red = *Choloramfenicol* Green

This is in accordance with Hamzah (2017) statement that interactions in biofilm bacteria can improve the ability of other bacterial species to survive and grow in aquatic environments that trigger rapid responses and result in complex biofilm formation.

Effect of Thymol and Eugenol against polimicrobial biofilm phase eradication

In polymicrobial biofilm *P. aeruginosa* rapidly triggered *E. coli* growth, making it possible to form a boarder biofilm area. The interaction with biofilm bacteria such as *P. aeruginosa* increased the ability of *E. coli* to survive and grow in an aquatic environment. In polymicrobial biofilm, they were well-organized in order to ful fill each others' nutrition. We reported that thymol and eugenol degraded 50% formation of *C. albicans*, *P. aeruginosa*, *E. coli* and *S. aureus* biofilm (Figure 8).

It was harder to damage the microbes that formed biofilm compared to microbes that formed biofilm in a middle and inhibition phase. This caused by the longer growth time of biofilm, lead toformation of strong bacteria community so that antibiotics are difficult to penetrate. Matrices extracellular polymer subtances (EPS) in biofilms may help cells survive longer than if a set of conditions because EPS bound with water in an abundance amount so the microbes form a strong biofilm that had resistance toward drought, nutrients' deficiency, anti-microbial substance and other conditions that brought disadvantages to the microbes' growth. The EPS matrix on biofilms can help microorganism cells last longer than when they are in planktonic conditions, so it can help to microcortium various species of microorganisms in the degradation process (Donlan, 2002).

In this study, we reported that thymol and eugenol compound had activity of biofilm damaging. The $MBEC_{50}$ value of thymol and eugenol are 0.17 and 0.24 *(*P<0.05)* respectively. According to this study, it can be reported that the inhibition activities of thymol and eugenol compound in biofilm degradation phase were more potent compared to the control such as nystatin 56.66 % ± 0.96 *%;* while in chloramphenicol, the inhibition activitiesof thymol and eugenol compound in biofilm degradation phase were found almost similar 72.63±3.33%.

Therefore effectiveness of thymol and eugenol against polymicrobial biofilm is an interesting thing to the discovery of new antibiofilm compounds as a therapeutic option for biofilm polymicrobial related infections.

CONCLUSION

Thymol and Eugenol potential as potential sources of novel antibiofilm candidates against polymicrobial biofilms *C. albicans – P. aeruginosa - E. coli* and *S. aureus*.

ACKNOWLEDGEMENT

Authors acknowledge Faculty of Pharmacy UGM for research financial support.

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